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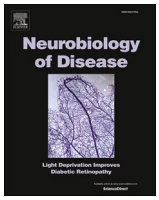

# HCN channels and absence seizures

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ABSTRACT

Hyperpolarization-activation cyclic nucleotide-gated (HCN) channels were for the first time implicated in absence seizures (ASs) when an abnormal $I_h$ (the current generated by these channels) was reported in neocortical layer 5 neurons of a mouse model. Genetic studies of large cohorts of children with Childhood Absence Epilepsy (where ASs are the only clinical symptom) have identified only 3 variants in *HCN1* (one of the genes that code for the 4 HCN channel isoforms, HCN1–4), with one (R590Q) mutation leading to loss-of-function. Due to the multi-faceted effects that HCN channels exert on cellular excitability and neuronal network dynamics as well as their modulation by environmental factors, it has been difficult to identify the detailed mechanism by which different HCN isoforms modulate ASs. In this review, we systematically and critically analyze evidence from established AS models and normal non-epileptic animals with area- and time-selective ablation of *HCN1*, *HCN2* and *HCN4*. Notably, whereas knockout of rat *HCN1* and mouse *HCN2* leads to the expression of ASs, the pharmacological block of all HCN channel isoforms abolishes genetically determined ASs. These seemingly contradictory results could be reconciled by taking into account the well-known opposite effects of $I_h$ on cellular excitability and network function. Whereas existing evidence from mouse and rat AS models indicates that pan-HCN blockers may provide a novel approach for the treatment of human ASs, the development of HCN isoform-selective drugs would greatly contribute to current research on the role for these channels in ASs generation and maintenance as well as offer new potential clinical applications.

## 1. Introduction

Absence seizures (ASs) are a type of genetic generalized seizures that are characterized by relatively brief loss of consciousness and electroencephalographic 2.5–4 Hz spike-and-wave discharges (SWDs) (Blumenfeld, 2005; Crunelli and Leresche, 2002). These seizures are highly prevalent in children and teenagers and the only clinical symptom in Childhood Absence Epilepsy (CAE), but are also present, together with other seizure types, in various adult epilepsies (Scheffer et al., 2017). Studies in both CAE cohorts and well-validated models of ASs have now conclusively demonstrated that the SWDs of typical ASs result from the aberrant behaviour of corticothalamic (CT) networks and are modulated by the basal ganglia (Cavarec et al., 2019; Crunelli et al., 2020; Deransart and Depaulis, 2002). Notwithstanding, the molecular, cellular and network mechanisms underlying the SWDs of ASs are still not fully understood: thus, knowledge of the contribution of HCN channels to AS epileptogenesis and ictogenesis would contribute to the identifications of novel targets for treating the 30% of CAE children who are pharmaco-resistant (Cnaan et al., 2017) and the 60% who show neuropsychiatric comorbidities that are insensitive to current anti-absence medicines (Masur et al., 2013).

Hyperpolarization-activation cyclic nucleotide-gated (HCN)

*Abbreviations:* CAE, Childhood Absence Epilepsy; CIN, cortical initiation network; CT, corticothalamic; ETX, ethosuximide; HCN, hyperpolarization-activated cyclic nucleotide-gated non-specific cation channel; KD, knock-down; KO, knock-out; NRT, nucleus reticularis thalami; $R_N$, input resistance; S1, primary somatosensory cortex; SWD, spike-and wave discharge; TC, thalamocortical; TRP-8b, TRP-containing RAb8b-interacting protein; $V_{½}$, half-voltage of activation; VB, ventrobasal thalamic complex; $V_m$, resting membrane potential.

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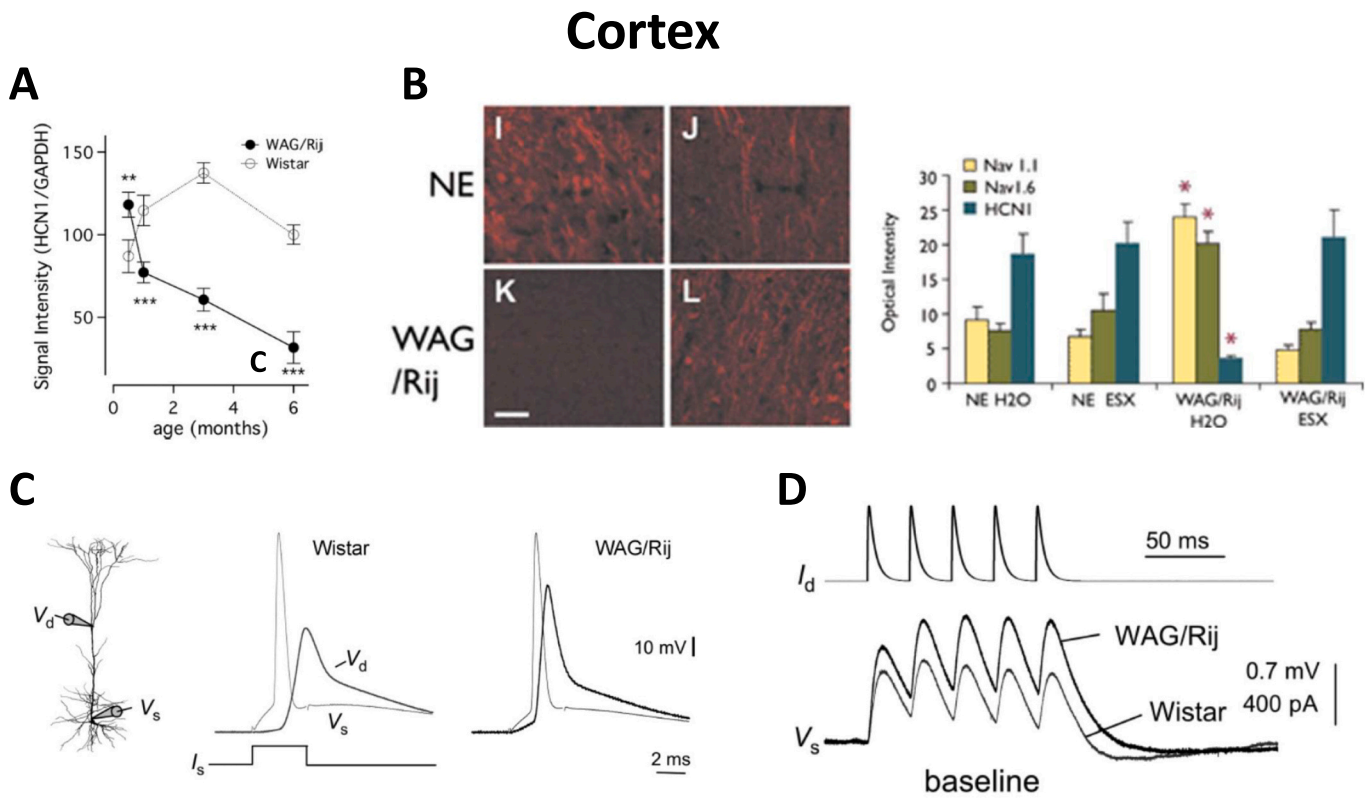


**Fig. 1.** HCN channel levels and function in the primary somatosensory cortex of WAG/Rij rats.
A) Postnatal development of the expression levels of HCN1 channels in S1 of Wistar and WAG/Rij rats. B) Left: HCN1 immunostaining in S1 of non-epileptic (NE) and WAG/Rij rats drinking normal water alone (H2O) (left pictures) or with ethosuximide (ETX) (right pictures) from postnatal day 21 for 5 months. Right: quantification of immunostaining of HCN1, Nav1.1 and Nav1.6 proteins in the rat strains drinking water or water+ETX. C) Dendritic and somatic recordings in S1 layer 5 pyramidal neurons showing somatic ($V_s$) and dendritic ($V_d$) action potentials in Wistar and WAG/Rij rats. Is: somatic current injection. D) Summation ($V_s$) of dendritically injected artificial EPSPs ($I_d$) in Wistar and WAG/Rij rats. Modified from Kole et al. (2007) (A, C, and D) and Blumenfeld et al. (2008) (B).

channels are present throughout the CNS (Notomi and Shigemoto, 2004) and display region-, cellular- and subcellular-specific differences in the relative expression of their four isoforms (HCN1 to HCN4) (Santoro and Shah, 2020). HCN channels play a crucial role in modulating neuronal excitability, synaptic integration and thus network output (Biel et al., 2009; Combe and Gasparini, 2021), and are known to play a key role in different forms of convulsive seizures, including generalized tonic-clonic seizures (Shah et al., 2013) and febrile seizures (Wenzel et al., 2023).

In this review, we first provide a short summary of the main genetic and pathophysiological features of ASs in humans and animal models, and then present an outline of the CNS distribution of HCN channels and their role in neuronal excitability and network function. Using data from animal models of ASs and genetic alterations in normal non-epileptic animals, the main body of this review is a critical discussion of the established and potential mechanisms by which HCN channels of different neuronal populations in CT networks contribute to the generation and maintenance of these seizures. We conclude with a summary of the still unanswered questions concerning the contribution of HCN channels to ASs to help guide future research in this field. We have aimed to provide sufficient details on ASs and HCN channels, so that our critical presentation and conclusions could be easily followed by the non-experts in the respective fields. Notwithstanding, comprehensive reviews on ASs (Blumenfeld, 2005; Crunelli et al., 2020; Lüttjohann and van Luijtelaar, 2022) and HCN channels (Combe and Gasparini, 2021; Kessi et al., 2022; Santoro and Shah, 2020) are available.

## 2. Absence seizures

Though ASs are classified as genetic generalized seizures, it has now been conclusively demonstrated that they are not generalized from the start since their SWDs (and the fMRI BOLD signal that they generate) originate from a localized cortical region (the cortical initiation network, CIN) before they fully generalize to the rest of the neocortex and the thalamus (Bai et al., 2010; Guo et al., 2016). In children with CAE, the CIN is preferentially located in frontal and fronto-parietal regions, may differ from child to child but is consistent between different seizures in the same child (Bai et al., 2010; Guo et al., 2016).

Human ASs have a unique pharmacological profile among seizure types: they are blocked by ethosuximide (ETX), valproate and to a lesser extent by lamotrigine and aggravated by carbamazepine, vigabatrin and tiagabine. This, together with other specific AS features, makes it imperative for studies on their mechanisms to focus exclusively on subjects with CAE since other pediatric and juvenile genetic epilepsies include other seizure types that may affect the expression and maintenance of ASs (Crunelli et al., 2020).

Although many spontaneous single-gene mutations in mice can lead to the expression of SWDs, in this review we will only consider data from well-validated models of ASs: the Wistar-Albino-Glaxo from Rijswijk (WAG/Rij) and Genetic Absence Epilepsy Rats from Strasbourg (GAERS) rats models and the monogenic stargazer (STG), lethargic (LH) and tottering (TG) mouse models (Crunelli and Leresche, 2002). These models well reproduce the CAE clinical and electrographic features and their pharmacological profile (*i.e.* block by ETX, valproate and lamotrigine and aggravation by vigabatrin, tiagabine and carbamazepine), the critical role of CT networks in seizure generation and maintenance and the existence of a CIN. Notably, before being recognized in children with CAE, the CIN was originally identified in genetic rat models (Meeren et al., 2002), where it is located in the primary somatosensory

**Table 1**
Cortical and thalamic HCN expression levels in genetic models of absence seizures.

| Cortical HCN protein levels | | | | Thalamic HCN protein levels | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| WAG/Rij | | | | WAG/Rij | | | | GAERS | | | |
| HCN Isoform | Age | Area | Reference | HCN Isoform | Age | Area | Reference. | HCN Isoform | Age | Area | Reference |
| ↑ HCN1<br>↓ HCN1<br>= HCN2 | P15<br>P30-180<br>P15-180 | S1 | Kole et al, 2007 | ↑ HCN1<br>= HCN2-4 | NI | dLGN | Budde et al, 2005 | ↑ HCN1<br>↑ HCN3 | P120-150<br>P120-150 | VB | Cain et al, 2015 |
| ↓ HCN1<br>= HCN2-4 | P70-90 | S1 | Strauss et al, 2004 | = HCN1<br>↑ HCN1<br>= HCN2,4<br>↑ HCN3<br>= HCN3 | P7-14<br>P30-90<br>P7-90<br>P7<br>P14-90 | dLGN | Kanyshkova et al, 2012 | ↑ HCN1#<br>= HCN2,4#<br>#(mRNA) | NI | VB & NRT | Kuisle et al, 2006 |
| ↓ HCN1<br>= HCN2-4 | Adult | NI | Blumenfeld et al, 2008 | | | | | | | | |

P: postnatal day; S1: primary somatosensory cortex; dLGN: lateral geniculate nucleus; VB: ventrobasal thalamic complex; NI: not identified; ↓: decrease; ↑: increase; =: no change.

cortex (S1) (mainly the peri-oral region), with layer 5 pyramidal neurons being the first cortical neuronal population to show ictal bursting activity (Polack et al., 2007). Thus, to understand the contribution of HCN (or any other channels) to ASs, it is of paramount importance to study S1 of genetic models and its somatotopic thalamic nuclei, the ventrobasal (VB) thalamic complex, an approach that, however, has regrettably not always been followed in studies investigating the contribution of HCN channels to ASs. The only other differences between children with CAE and AS models are the higher frequency of SWDs (5–6 and 7–8 Hz in mouse and rat models, respectively) and the lack of AS age-dependent remittance since ASs continue to be present during adulthood in animal models.

## 3. HCN channels

HCN channels are voltage-gated channels that open in response to membrane hyperpolarization from membrane potentials $\leq$ -55 mV, allowing an increased flow of $Na^+$ and $K^+$ ions. Four genes (*HCN1*, *HCN2*, *HCN3* and *HCN4*) encode the 4 isoforms of HCN channels that consists of a tetramer of subunits, each with a six-transmembrane topology (Combe and Gasparini, 2021). HCN channels are generally homomeric channels but can interact to form functional heteromeric channels, leading to different properties of the inward current ($I_h$) that they generate. Thus, for example, the $I_h$ of HCN1 homomeric channels has a rapid activation kinetics whereas that of HCN2 and HCN4 has slower activation. The sensitivity to cAMP is a striking feature of HCN channels (since it allows them to integrate neuronal activity with neuromodulatory inputs) and contributes to the regulation of excitability and synaptic plasticity in a variety of brain regions and is greater for HCN4 and HCN2 and much smaller for HCN1 channel (Combe and Gasparini, 2021). In addition, phosphorylation and glycosylation affect not only the number of HCN channels expressed on the plasma membrane but also their heteromeric assembly.

The location of HCN channels varies within different neuronal populations and their subcellular compartments (Lai and Jan, 2006; Narayanan and Johnston, 2008; Nusser, 2012). The dendritic predominance of HCN channels in neocortical pyramidal neurons (see below) is in part under the control of the accessory protein TRP-containing RAb8b-interacting protein (TRP-8b) that binds to their C-terminal increasing their plasma membrane expression (Lewis et al., 2009; Santoro et al., 2009). HCN1 channels (but not HCN2–4) interact with filamin A (a scaffold protein with actin-binding domains) in a region of the C-terminal downstream from the cAMP-binding domain, localizing this HCN isoform in hot spots on the plasma membrane (Gravante et al., 2004). HCN1 is abundantly expressed in the neocortex, hippocampus, cerebellum and brainstem (Moosmang et al., 1999; Notomi and Shigemoto, 2004), HCN2 subunits are globally distributed through the CNS, with high expression levels in the thalamus, HCN3 subunits show a low CNS expression and HCN4 subunits are found in selective brain regions, such as the thalamus and the olfactory bulb but at lower levels than HCN2 (Moosmang et al., 1999; Notomi and Shigemoto, 2004).

HCN channels are highly expressed on dendrites but less on the soma of neocortical pyramidal neurons: indeed their gradient distribution along the somatodendritic arbor shows a 10-fold higher density in the distal dendrites than in the soma (Lörincz et al., 2002; Magee, 1998). HCN2 levels are ten-fold higher in the soma of TC than NRT neurons, while HCN4 show a similar expression in the two neuronal types (Abbas et al., 2006). Notably, HCN2 is the predominant isoform in NRT neurons and is principally located in dendritic spines (Abbas et al., 2006) where it co-localizes with the mGluR4 subunit (Ying et al., 2007). Moreover, the co-localization of HCN2 and HCN4 channels is higher in TC than in NRT neurons (Abbas et al., 2006).

As voltage-gated ion channels that open in a voltage region around the resting membrane potential, HCN channels play a fundamental role in the regulation of cellular excitability, synaptic integration and neuronal network dynamics. Notably, HCN channels exert opposite effects on neuronal function since their opening increases excitability by depolarizing the membrane potential but concomitantly dampens excitability by decreasing membrane resistance. In neocortical pyramidal neurons, because of their differential distribution in the somatodendritic axis, HCN channels tightly control the summation of dendritic EPSPs, so that their block augments dendritic integration despite a hyperpolarized membrane potential (Magee, 1998, 1999; Berger et al., 2001; Poolos et al., 2002; Shah et al., 2004; Huang et al., 2009). This, in turn, leads to larger and slower EPSPs, leading to an enhanced somatic summation of EPSPs and ultimately boosting neuronal excitability (Shah et al., 2004). Moreover, the activation of $I_h$ during trains of IPSPs reduces synaptic hyperpolarization (Williams and Stuart, 2003; Atherton et al., 2010; Pavlov et al., 2011), thus limiting the de-inactivation of the current generated by T-type $Ca^{2+}$ channels ($I_T$) and rebound firing

**Table 2**
$I_h$ and membrane properties of cortical and thalamic neurons in genetic models of absence seizures.

| Cortical neurons *in vitro* | | | | | | | Thalamocortical neurons *in vitro* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WAG/Rij | | | | STG | | | WAG/Rij | | | | GAERS | | | |
| | Area & cell type | Age | Refer. | | Area & cell type | Age | Refer. | | Area | Age | Refer. | | Area | Age | Refer. |
| ↓ $I_h$ (dendrites)<br>= $I_h$ $V_{1/2}$<br>↓ channel number<br>= single channel conduc.<br>↑ dendritic summation<br>↑ backpropagating AP<br>↑ bursting | S1 L5 pyramid | 3-5 m | Kole et al, 2007 | ↑ $I_h$<br>↑ depolar sag | NI L5/6 pyramid | 3 w<br>3 m | Di Pasquale et al, 1997 | = $I_h$<br>↓ τ activation<br>$I_h$ $V_{1/2}$ hyperpolar (7 mV)<br>↓ c-AMP-modulation<br>↓ burst to tonic shift<br><br>$I_h$ $V_{1/2}$ hyperpolar (3 mV) | dLGN<br><br>VB | 2 w & 1 m | Budde et al, 2005 | ↑ $I_h$ (-110 to -60 mV)<br>↑ $I_h$ (-110 to -90 mV)<br>= $I_h$ (-80 to -60 m V)<br>= $V_{1/2}$<br>= bursting<br>↓ bursting<br>= $V_m$<br>↓ $R_n$ | VB | 2-3 w<br>4-5 m<br>4-5 m<br>2 w & 5 m<br>2-3 w<br>4-5 m<br>2 w & 5 m<br>2 w & 5 m | Cain et al, 2015 |
| ↓ $I_h$ (soma)<br>$I_h$ $V_{1/2}$ depolar (9 mV)<br>↓ τ activation<br>↓ τ de-activation<br>↑ somatic summation<br>↑ bursting | S1 L2/3 pyramid | 2-3 m | Strauss et al, 2004 | | | | | ↑ $I_h$<br>↓ τ activation<br>$I_h$ $V_{1/2}$ hyperpolar (6 mV)<br>↓ c-AMP-modulation | dLGN | 2 w & 1 m | Kanyshkova et al, 2012 | = depolarizing sag<br>= $I_h$ amplitude<br>= $V_{1/2}$<br>= $R_n$<br><br>↓ c-AMP-modulation | VPM | 3-8 m<br><br>3 w & 8 m | Kuisle et al, 2006, |

$V_{1/2}$: half-maximal voltage of activation; pyramid: pyramidal neuron; S1: primary somatosensory cortex; L5: cortical layer 5; L2/3: cortical layer 2/3; NI: not identified cortical area; m: month; w: week; dLGN: dorsal lateral geniculate nucleus; VB: ventrobasal thalamic complex; VPM: ventroposteromedial nucleus; $V_m$: resting membrane potential; $R_n$: input resistance; ↓: decrease; ↑: increase; =: no change.

(Atherton et al., 2010). At the same time, the interaction of $I_h$ and $I_T$ does lead to pacemaker firing (consisting of rhythmic T-type $Ca^{2+}$ channel-mediated bursts), a feature particularly evident in thalamocortical (TC) and nucleus reticularis thalami (NRT) neurons (Leresche et al., 1991; McCormick and Pape, 1990). Notably, pharmacological block of $I_h$ (and *HCN2* deletion, see Section 7) markedly increases tonic single action potential firing in NRT neurons and enhances the number of large spontaneous IPSC in TC neurons (Ying et al., 2007).

Finally, HCN channels have been shown to act as a high-pass filter and thus increase the subthreshold resonance of neocortical and hippocampal neurons in the theta frequency band (Fransén et al., 2004; Narayanan and Johnston, 2008; Vaidya and Johnston, 2013), making them ideal candidates to control and modulate physiological and pathological oscillations of neuronal networks in this frequency band.

## 4. *HCN1–4* variants in CAE and models of ASs

The four human *HCN* genes are located on different chromosomes: *HCN1* in 5p12, *HCN2* in 19p13, *HCN3* in 1q21.2 and *HCN4* in 15q24-q25, a differential pattern of location that is maintained from fish through mammals (Jackson et al., 2007). Since the first report of changes in $I_h$ following febrile seizures in animal models (Chen et al., 2001), many studies have observed alterations in HCN expression and functions in other models of febrile seizures and temporal lobe epilepsy (mainly associated with hippocampal sclerosis) (Wenzel et al., 2023). This, in turn, prompted a search for *HCN1–4* variants in the genetic epilepsies, particularly those with generalized seizures.

A critical analysis of the available data indicates that whereas *HCN* mutations are detected in patients with genetic generalized epilepsy (GGE), their presence in patients experiencing only ASs, *i.e.* subjects with CAE, is not as common Thus, in a cohort of 585 GGE patients, the *HCN2* p.V246M variant was present in 3 subjects that had generalized SWDs accompanied by either generalized tonic-clonic seizures, prominent photosensitivity or centrotemporal spikes and mutations in *HCN1*, *HCN2* and *HCN4* were reported in patients with GGE who had ASs together with other more severe seizure types and moderate to severe intellectual disability (Campostrini et al., 2018; DiFrancesco et al., 2011; Li et al., 2018; Nava et al., 2014).

The only 3 patients where *HCN* variants are clearly linked to a CAE diagnosis had mutations in *HCN1* (Marini et al., 2018). The patient carrying the p.Arg590Gln variant exhibited pharmaco-resistant CAE with a single tonic-clonic seizure and an SWD morphology of typical ASs with frequency slowing, and no post-ictal depression. The functional analysis of this variant expressed as a homotetrameric channel, demonstrates a clear loss-of-function (Marini et al., 2018). The other two CAE patients had a p.Ser680Tyr variant (which is predicted to have a gain-of-function), and a p.Arg715Gly variant in *HCN1*, (the functional consequences of which are difficult to predict) respectively (A. Porro, C. Marini, B. Santoro, personal communication).

Moreover, in a genome-wide mega-analysis of >15,000 epileptic patients, significant loci were identified on chromosome 2 (2p16.1 and 2q22.3) in 793 CAE subjects (778 Caucasian, 15 African), where none of the *HCN1–4* genes are located (International League Against Epilepsy Consortium on Complex Epilepsies, 2018). Similarly, a whole-exome sequencing of >17,000 epileptic patients did not find any significant genetic variants in their large CAE cohort (Epi25 Collaborative, 2019).

As far as genetic AS models are concerned, whole-genome sequencing of the polygenic GAERS rats did not detect any *HCN1–4* variants but identified *CACNA1H* (R1584P) as the only mutation that significantly co-segregated with high-seizing F2 progenies (Casillas-Espinosa et al., 2017). A chromosomal mapping of WAG/Rij rats reported a significant QTL in chromosome 5 (where *HCN1* is located) (Gauguier et al., 2004). Notably, an N-terminal deletion variant of *HCN1* has been found in thalamic mRNA, but not in genomic DNA, of this polygenic rat model, leading to a 2.2-fold increase in $I_h$ amplitude when expressed in *Xenopus* oocytes (Wemhöner et al., 2015). Notably, in co-

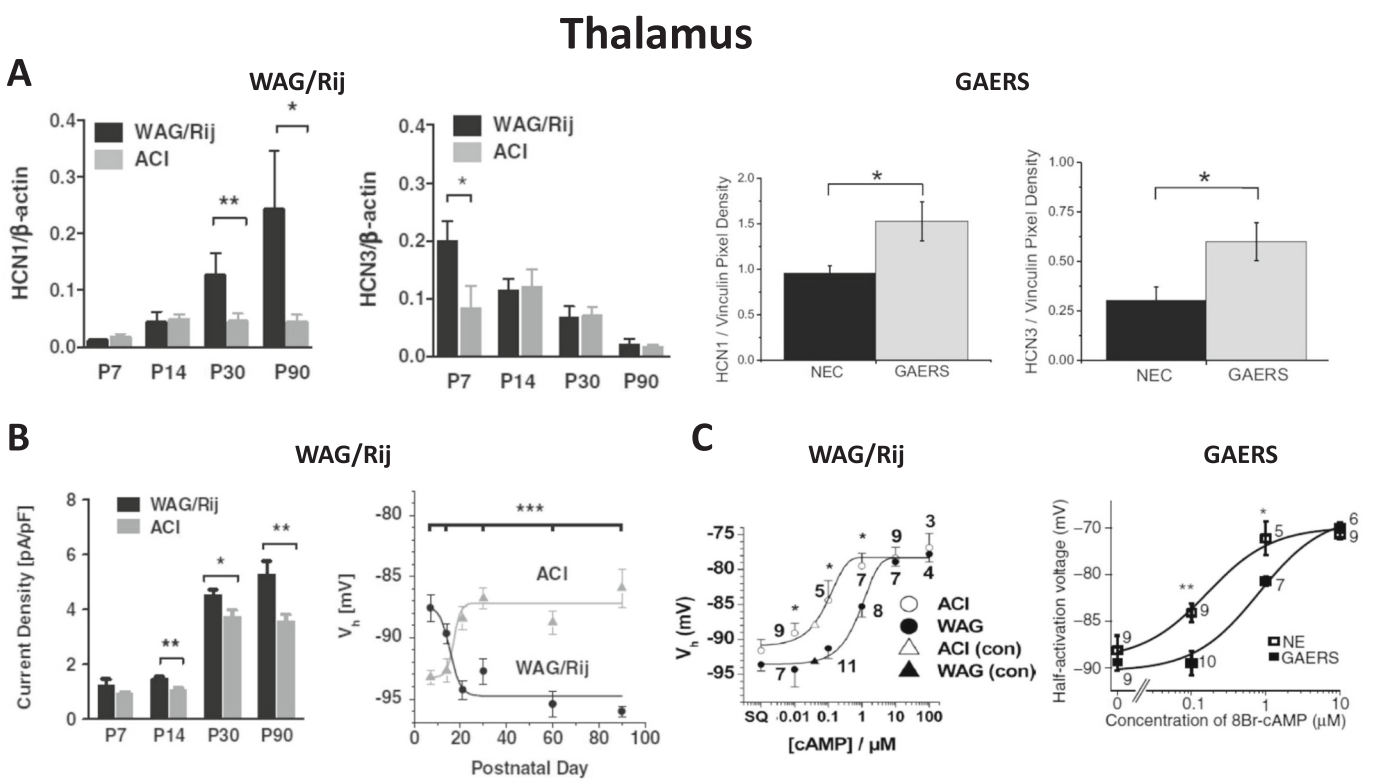


**Fig. 2.** HCN channel levels and function in TC neurons of the ventrobasal thalamic complex of GAERS and WAG/Rij rats.
A) HCN1 and HCN3 protein levels in developing WAG/Rij rats (left plots) and in adult GAERS rats (right plots) compared to non-epileptic control rat strains (ACI and NEC, respectively). B) Postnatal development of the density (left plot) and half-voltage of activation ($V_{½}$) (right plot) of $I_h$ in WAG/Rij and ACI rats. C) Dose-response curves of the cAMP-sensitivity of $I_h$ in adult WAG/Rij, GAERS and non-epileptic control rat strains (ACI and NEC, respectively). Modified from Kanyshkova et al. (2012) (A, B), Cain et al. (2015) (A), Kuisle et al. (2006) (C) and Budde et al. (2005) (C).

expression experiments, this *HCN1* variant reduces HCN2 and HCN4 currents and decreases cAMP-sensitivity of HCN2 channels, thus in part explaining the decreased cAMP-sensitivity observed in TC neurons of WAG/Rij rats (Table 2) (Kanyshkova et al., 2012).

In summary, only 3 *HCN1* variants have been detected in >2000 patients with CAE analyzed in different studies of American, Australian, European and African cohorts, but none in *HCN1–3*. The rare occurrence of human *HCN1–4* mutations mirrors the well-validated GAERS and WAG/Rij rat models, with only 1 thalamic mRNA variant in *HCN1* and none in *HCN2–4*. Notwithstanding, clear alterations in HCN channels expression and function are present in neocortical and thalamic territories of the polygenic rat models compared to normal non-epileptic animals, as detailed in the next section.

## 5. Abnormalities of HCN channel expression and function in AS models

At P15, HCN1 levels in the CIN are higher in WAG/Rij rats than in controls (Kole et al., 2007), whereas from 1 month till adulthood there is a decreased expression of HCN1 proteins, with no change in HCN2–4 (Fig. 1A) (Table 1) (Blumenfeld et al., 2008; Kole et al., 2007; Strauss et al., 2004). Chronic treatment with ETX from P21 for 5 months blocks ASs and normalizes HCN1 levels to those of non-epileptic rats (Blumenfeld et al., 2008) (Fig. 1B). The link between the normalization of HCN1 expression and the rescue of ASs by chronic ETX remains to be established, since the levels of Nav1.1 and Nav1.6 channel isoforms were also normalized to those of control rats (Fig. 1B) (Blumenfeld et al., 2008). Feeding WAG/Rij mothers with a methyl-enriched diet from 5 days prior to gestation to 1 week after parturition decreases ASs occurrence and increases HCN1 levels in the CIN of their adult progenies (Sarkisova et al., 2023). However, a causal link between this diet, changes in HCN1 levels, and ASs remains to be demonstrated.

The cellular consequences of the decreased HCN1 channel expression in WAG/Rij rats were investigated *in vitro* in layer 2/3 neurons (Strauss et al., 2004) and later with simultaneous dendritic and somatic recordings in S1 layer 5 pyramidal neurons (Kole et al., 2007), the CIN of WAG/Rij rats (Table 2). These studies showed that these S1 pyramidal neurons have a markedly reduced dendritic $I_h$ which, in turn, increases somato-dendritic coupling and reduces the frequency-threshold for the generation of dendritic $Ca^{2+}$ spikes by backpropagating action potentials and the summation of depolarizing synaptic potentials (Fig. 1C, D). The overall result of these changes is a higher probability of burst firing, a finding similar to that observed *in vivo* in layer 5 neurons of the CIN in GAERS rats (Polack et al., 2007). Notably, Di Pasquale et al. (1997) reported an increased depolarizing sag and a larger $I_h$ in layer 5 pyramidal neurons in a not-identified cortical area of STG mice (Table 2), but did not study potential changes in cortical HCN expression. Whether an enhanced cortical $I_h$ is also a feature of other single-mutation mouse models of ASs, *i.e.* LH and TG, that thus differentiates their cortical HCN channel function from that of the polygenic WAG/Rij rats, remains to be established. Regrettably, there is no data on HCN expression levels and function in the CIN of GAERS rats.

In contrast to what is found in the neocortex, there is a progressive developmental increase in HCN1 (but not HCN2 and 4) proteins in TC neurons of WAG/Rij rats that becomes significant at P30 and P90 compared to non-epileptic rats (Fig. 2A) (Table 1) (Kanyshkova et al., 2012). A similar increase in HCN1 (but not HCN2 and 4) proteins is observed in TC neurons of adult GAERS rats (Cain et al., 2015; see also Kuisle et al., 2006) (together with an increase in HCN3 levels) and only at P7 in WAG/Rij rats (Fig. 2A) (Table 1).

A similar increase in HCN1 levels (with no change in the HCN 2 and 4 isoforms) has been found in GAERS NRT neurons (Table 1) (Kuisle et al.,

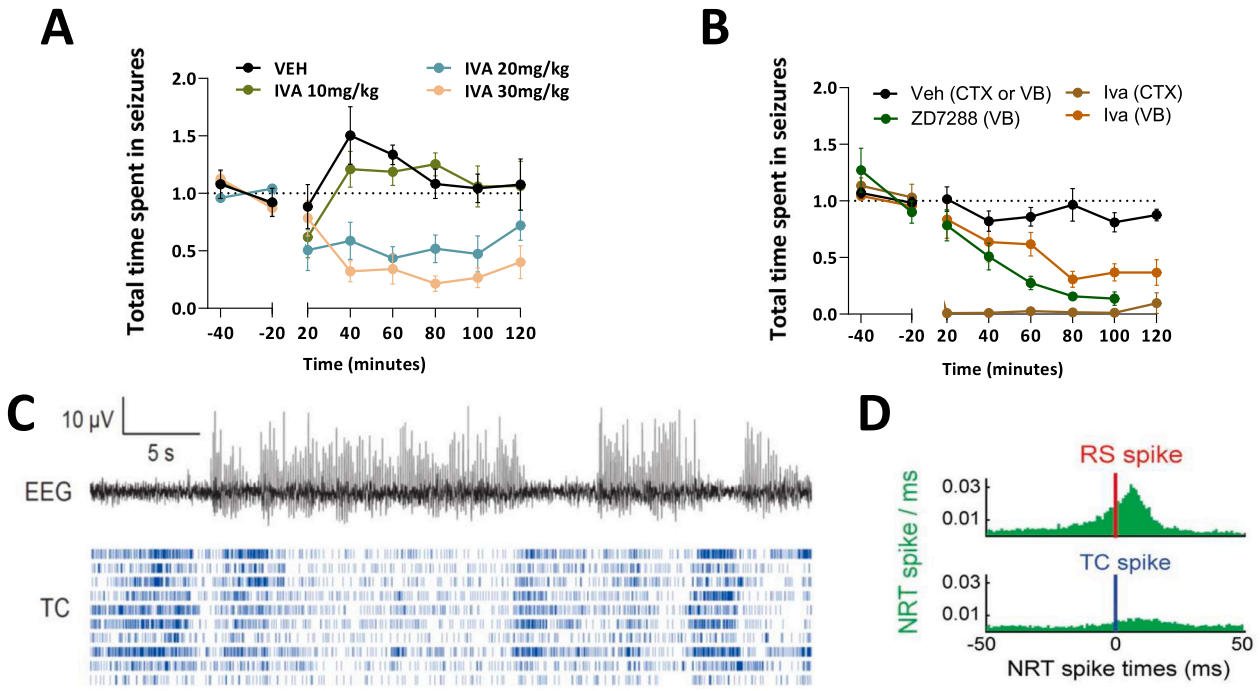


**Fig. 3.** Effect of pan-HCN channel blockers on absence seizures and ictal activity of TC and NRT neurons in freely moving GAERS rats.
A) Dose-dependent block of spontaneous ASs in GAERS rats by systemic administration of ivabradine at time 0. Elacridar, a phosphoglycoprotein inhibitor, was injected 20 min prior to ivabradine and had no effect on its own on ASs (not shown). B) Block of AS by cortical injection of ivabradine and thalamic injection of ivabradine and ZD7288. C) EEG (top trace) and raster-plot of the interictal and ictal activity of VB TC neurons during spontaneous ASs in a freely moving GAERS rat. D) Plot of ictal action potential firing in an NRT neuron during ASs. The 0 time-point refers to the firing of simultaneously recorded cortical layer 5 regular spiking neurons (red bar) and TC neurons (blue bar). Modified from Iacone et al. (2021) (A, B), David et al. (2018) and McCafferty et al. (2018) (C, D).

2006). The increased expression of HCN1 (and potentially HCN3) brings about a larger $I_h$ of TC neurons in young (P14) and adult WAG/Rij and young (P15–20), but not adult GAERS rats (Fig. 2B) (Table 2) (Cain et al., 2015; Kanyshkova et al., 2012; Kuisle et al., 2006). Moreover, $I_h$ voltage-threshold of activation is hyperpolarized in WAG/Rij (but not in GAERS) rats (Fig. 2B) (Table 2) (Budde et al., 2005; Cain et al., 2015; Kanyshkova et al., 2012; Kuisle et al., 2006), and the sensitivity of the current to cAMP is decreased in both rat models (Fig. 2C) (Table 2) (Budde et al., 2005; Kanyshkova et al., 2012; Kuisle et al., 2006). Regrettably, there are no data on $I_h$ and the expression of HCN channel isoforms in thalamic neurons of STG, LH and TG mice.

The cellular consequences of these changes in the $I_h$ of TC neurons have been studied either in anaesthetized animals or *in vitro* by mainly investigating the contribution of this current to the generation of spontaneous or evoked T-type $Ca^{2+}$ channel-dependent burst firing. However, recent findings in freely moving GAERS rats challenge the idea that this type of firing is a significant contributor to the ictogenic activity of ASs (McCafferty et al., 2018, 2023). Therefore, we have not included the results of these studies in Table 2 and thoroughly discussed this issue in Section 9.

In summary, 1) in the neocortex, there is a lower expression of HCN1 in WAG/Rij (though, notably, HCN1 levels are higher at P15), whereas there is no change in HCN2–4 expression; 2) in the thalamus of both WAG/Rij and GAERS rats there is an increased expression of HCN1 (and possibly HCN3) whereas no change is observed in the HCN4 and HCN2 isoforms (the most abundantly expressed isoforms in this brain region); and 3) exogenous manipulation (either by chronic treatment with ETX or by a methyl-enriched diet of pregnant mothers) rescues the aberrantly lower cortical HCN1 levels of WAG/Rij rats, though the causal link between $I_h$ functional changes induced by these procedures and the block of ASs remains to be established.

## 6. Effect of blocking HCN channels in AS models

Application of the pan-HCN channel blocker, ZD7288, by microdialysis in the VB of freely moving animals, was shown to markedly decrease genetically determined ASs in GAERS rats (Fig. 3B) as well as ASs elicited by systemic injection of γ-hydroxybutyric acid (GHB) (David et al., 2018). A recent study (Iacone et al., 2021) evaluated the efficacy of ivabradine, another pan-HCN channel blocker approved for heart failure treatment in humans (Savelieva and Camm, 2006), against ASs in GAERS. Systemic administration of ivabradine effectively blocked ASs in GAERS rats in a dose-dependent manner (Fig. 3A). Moreover, its administration into the CIN of GAERS rats was highly effective in rapidly eliminating ASs, while its anti-absence effect was slower and less potent when it was injected into the VB (Fig. 3B) (Iacone et al., 2021). Furthermore, injection of a pan-HCN channel-blocking *sh*RNA in the VB of freely moving STG mice reduced their spontaneous ASs (David et al., 2018). The impact of HCN blockers on the spontaneous ASs of WAG/Rij rats and LH and TG mice has not yet been explored.

At present, there is only one drug, EC18, that selectively blocks one HCN channel isoform. This drug has a six-fold higher selectivity for HCN4 compared to HCN1 and HCN2 channels and a similar potency on HCN4 channels than ivabradine (Romanelli et al., 2019). EC18 abolishes the $I_h$ of VB TC neurons in brain slices but also reduces the sustained, but not the transient, outward $K^+$ currents of these neurons (Romanelli et al., 2019). Systemic injection of EC18 decreases convulsive seizure threshold and cortical spiking (elicited by pentylenetetrazol or kainic acid) (Kharouf et al., 2020), an effect that is occluded in adult mice with conditional brain-specific KO of HCN4 channels (Kharouf et al., 2020). The effect of EC18 on pharmacologically induced or genetically determined ASs has not been tested.

Currently, there is no drug that selectively enhances HCN channel function. Lamotrigine, an anti-absence medication (Brigo et al., 2021), increases $I_h$ in animal and human hippocampal and cortical neurons

**Table 3**
Effects of genetic manipulation of HCN isoforms in normal non-epileptic animals.

| HCN isoform | Type & site of genetic manipulation | Spontaneous ASs (frequency) (duration) | Cortical HCN levels | Thalamic HCN levels | Properties of cortical neurons *in vitro* | Properties of thalamic neurons *in vitro** | Notes | Reference |
|---|---|---|---|---|---|---|---|---|
| HCN1 | WB constitutive KO | No | HCN1 absent<br>= HCN2,4<br>↑ HCN3 | HCN1 absent<br>= HCN2,4<br>↑ HCN3 | $I_h$ absent<br>↑ synaptic summation<br>(EC L3 pyr) | NT | Sensitivity to drug-induced ASs NT<br>↑ sensitivity to convulsive seizures | Santoro et al, 2010<br>Huang et al, 2009<br>Nolan et al, 2003 |
| HCN 1<br>(*rat*) | WB constitutive KO | Yes<br>(7-9 Hz) (2-5 s)<br>(ETX-sensitive) | NT | NT | ↓ depolariz. sag (L5 pyr) | NT | Founder rat strain (F344) reported to have spontaneous ASs<br>↑ sensitivity to convulsive seizures | Nishitani et al, 2019 |
| HCN2 | WB constitutive KO | Yes<br>(5 Hz) (2-3 s)<br>(ETX-sensitive) | HCN2 absent<br>= HCN1, 3, 4 | HCN2 absent<br>= HCN1, 3, 4 | NT | TC neurons<br>↓ $I_h$ (95% at -80 mV)<br>$V_m$ hyperpolarized (12 mV)<br>↑ burst firing at $V_m$<br>↑ large spontaneous IPSCs<br><br>NRT neurons<br>$I_h$ absent<br>↑ tonic, single AP firing<br>↑ EPSCs summation | Phenotype includes sinus dysrhythmia, body tremor and ataxia | Ludwig et al, 2003<br>Ying et al, 2007 |
| HCN2 | WB constitutive KO of cAMP-modulation# | Yes<br>(3-5 Hz) (4-5 s)<br>(ETX-sensitivity NT) | NT | = HCN2 | NT | TC neurons<br>= $I_h$ density<br>↓ cAMP-modulation<br>$V_m$ hyperpolarized (5 mV)<br>↑ burst firing at $V_m$ | No body tremor or ataxia | Hammelmann et al, 2019 |
| HCN2 | VB constitutive KO | Yes<br>(3-5 Hz) (4-5 s)<br>(ETX-sensitivity NT) | NT | NT | NT | NT | | Hammelmann et al, 2019 |
| HCN2 | VB viral KD in adults | Yes<br>(5 Hz) (2-3 s)<br>(ETX-sensitivity NT) | NT | NT | NT | NT | | Hammelmann et al, 2019 |
| Spontaneous *HCN2* mutation | 4 base-pair (TTCA) insertion | Yes<br>(3-7 Hz) (1-3 s)<br>(ETX-sensitive) | NT | HCN2 absent<br>= HCN1, 4 | NT | NT | ↑ sensitivity to convulsive seizures | Chung et al, 2009 |
| HCN4 | WB constitutive KO | No | HCN4 absent<br>= HCN1-3 | HCN4 absent<br>= HCN1-3 | = $I_h$<br>(S1 L5 pyr) | TC neurons<br>↓ $I_h$ (58% at -80 mV)<br>= cAMP-modulation<br>$V_m$ hyperpolarized (9 mV)<br>↑ $R_n$<br>↓ burst firing | | Zobeiri et al, 2019 |
| HCN4 | VB viral KD in adults | No | NT | NT | NT | NT | | Hammelmann et al, 2019 |
| HCN | WB constitutive KO of TRIP8b | Yes<br>(5 Hz) (1-2 s)<br>(ETX-sensitive) | ↓ HCN1-4 | ↓ HCN1-4 | ↓ depolariz. sag<br>= synaptic summation<br>(L5b PT pyr) | TC neurons<br>↓ $I_h$ density<br>↑ cAMP-modulation<br><br>NRT neurons<br>= $I_h$ | = cardiac function | Heuermann et al, 2016 |

All data in this table are from mice except those in the second line.
The effects of genetic alterations on spontaneous and evoked intrathalamically oscillations *in vitro* are not included since they do not reflect the ictal firing of thalamic neurons during ASs in freely moving animals (see Section 9).
#: mice carrying two distinct amino acid substitutions that remove cAMP-dependence of HCN2 function; ↓: decrease; ↑: increase; =: no change; NT: not tested; AP: action potential; EC L3 pyr: entorhinal cortex layer 3 pyramidal neurons; KO: knock-out; KD: knock-down; L5b PT pyr: layer 5b pyramidal tract neurons; L5 pyr: layer 5 pyramidal neurons; NRT: nucleus reticularis thalami; $R_n$: input resistance; S1 L5 pyr: layer 5 pyramidal neurons in primary somatosensory cortex; TC: thalamo-cortical; TRIP8b: tetratricopeptide-containing Rab8b-interacting protein; $V_m$: resting membrane potential.

(Poolos et al., 2002); Lehnhoff et al., 2019) but also affects many other voltage- and ligand-gated channels (Nakatani et al., 2013). Notwithstanding, the reduced dendritic firing and decreased synaptic summation elicited by lamotrigine in *in vitro* hippocampal neurons is mediated by HCN channels since it is fully blocked by the concomitant application of ZD7288 (Poolos et al., 2002). In clinic practice, lamotrigine monotherapy is less effective than ethosuximide (ETX) and valproate (~50 and 80% freedom from ASs, respectively) in treating children with CAE (Cnaan et al., 2017). This profile is similar to that of GAERS rats, where lamotrigine reduces ASs to a lesser extent than ETX and valproate (A. Depaulis, personal communication), and WAG/Rij rats, where lamotrigine inhibits SWDs only at high doses (van Rijn et al., 1994). Chronic treatment with lamotrigine reduces SWDs in Long Evans rats, but this strain is not yet fully validated as an AS model.

Gabapentin is another anti-seizure medicine that was originally reported to increase $I_h$ in hippocampal slices (Peng et al., 2011; Surges et al., 2013), but a more recent study showed that it selectively decreases HCN4 channel function (by hyperpolarizing their voltage-dependence of activation) in recombinant human HCN channels expressed in Xenopus oocytes and on native channels in spinal cord slices, with no effect on HCN1 and HCN2 (Tae et al., 2017). These contrasting results could be due to the lack of auxiliary proteins in the recombinant channels or to a different distribution of HCN channels in the studied tissue. Notwithstanding, due to the lack of data on the effect of gabapentin on the cortical and thalamic neurons responsible for AS generation, and its multiple effects on ligand- and other voltage-gated channels (Cheng and Chiou, 2006; Dolphin, 2016; Kelly, 1998; Stefani et al., 2001; Sutton et al., 2002) predicting its mechanism of action on ASs is challenging. Notably, Gabapentin has no effect on children with ASs (Schmidt, 1989) and is not used as an anti-absence medicine. Moreover, it worsens ASs in GAERS rats (Marescaux et al., 1992), but has not been tested in WAG/Rij rats or other AS models.

In summary, 1) there is strong evidence that systemic administration of pan-HCN channel blockers could be effective in abolishing human ASs; 2) blocking either cortical or thalamic HCN channels abolishes genetically determined ASs, with the cortex being more sensitive than the thalamus; 3) the potentiating effect of lamotrigine on HCN channel function is unlikely to be the key mechanism of its anti-absence action in humans and AS models; and 4) no HCN isoform-selective drug has been tested in mouse and rat genetic models of ASs.

## 7. AS-related effects of genetic manipulations of HCN channels in normal animals

Critical evidence on the role for HCN channels in ASs has been provided by studies where selective genetic alterations of their isoforms have been carried out in normal non-epileptic animals (Table 3). In both the cortex and thalamus of HCN1 constitutive KO mice, HCN1 proteins are absent whereas HCN2 and HCN4 expression is unchanged and HCN3 levels are higher compared to wild-type (WT) mice (Huang et al., 2009; Nolan et al., 2003; Santoro et al., 2010). Whole-brain and forebrain-selective HCN1 constitutive KO mice do not show spontaneous ASs (though their sensitivity to drug-induced ASs has not been tested) but have a higher susceptibility in acute and chronic models of convulsive seizures (Santoro et al., 2010). As expected from a reduced $I_h$, there is an increased dendritic integration of EPSPs in CA1 hippocampal neurons and layer 3 entorhinal cortex pyramidal neurons of HCN1 KO mice (Huang et al., 2009; Nolan et al., 2004; Santoro et al., 2010). Surprisingly, however, synaptic integration in S1 layer 5 pyramidal neurons is unaltered due to a compensatory increase in tonic $GABA_A$ current, which in turn depends on the enhanced expression of the $GABA_A$ α5 subunit and not by a loss-of-function of GAT1, one of the GABA transporters (Chen et al., 2010). This normalizes the EPSPs summation and may be a reason why HCN KO mice do not exhibit ASs. In contrast to mice, HCN1 KO rats do exhibit ETX-sensitive ASs and a decreased depolarizing sag of L5 pyramidal neurons but synaptic summation and HCN protein expression in the cortex and thalamus were not investigated (Table 3) (Nishitani et al., 2019). Moreover, the genetic background of the F344 rats used in this study might confound these results since this rat strain has spontaneous ASs (Buzsáki et al., 1990) that are blocked by ETX (Shaw, 2007). Finally, no data is available on thalamic neuron properties of both HCN1 KO mice and rats.

HCN2 constitutive KO mice do express spontaneous ETX-sensitive ASs (Ludwig et al., 2003) as do KD mice where the cAMP-modulation of HCN2 channels was genetically removed, mice with VB-selective constitutive KO of this HCN channel isoform and adult mice where HCN2 channels were virally deleted (Table 3) (Hammelmann et al., 2019). TC neurons in the VB of HCN2 KO mice have an almost absent $I_h$, at hyperpolarized $V_m$, enhanced burst firing at $V_m$ and increase in the number of large spontaneous IPSCs (Ludwig et al., 2003; Ying et al., 2007). NRT neurons of these mice show no $I_h$, an increase in tonic single action potential firing and an enhanced summation of EPSCs (Ying et al., 2007). TC neurons of mice with genetic KD of the cAMP-modulation had $I_h$-related properties similar to those of the HCN2 constitutive KO mice, though in this case $I_h$ density was unchanged (Hammelmann et al., 2019). No data were obtained for TC neurons in the other two HCN2 genetically altered mice, and there is a lack of data on cortical neurons for all four types of HCN2 mutant mice. HCN2 KO mice did not express HCN2 proteins but did not show any difference in the expression of HCN1, 3 and 4. Notably, a spontaneous mutation of *HCN2* in mice, that introduces a stop codon, results in no HCN2 channel expression in the thalamus and leads to infrequent ETX-sensitive ASs and ataxia (Table 3) (Chung et al., 2009).

In contrast, no ASs is observed in mice with brain-selective KO of HCN4 channels, that have no HCN4 proteins in both cortex and thalamus and similar levels of HCN1–3 (Table 3). The VB TC neurons of these mutants have a massive reduction in $I_h$, hyperpolarized $V_m$, increased $R_n$ and decreased burst firing but unchanged c-AMP-sensitivity, whereas $I_h$ of S1 layer 5 pyramidal neurons is unchanged (Zobeiri et al., 2019). Moreover, VB-selective KD of HCN4 channels does not lead to the expression of spontaneous ASs, but no data is available on the expression of HCN isoforms or neuronal properties in thalamus and cortex (Table 3) (Hammelmann et al., 2019). Notably, mice with constitutive KO of TRIP8b show ETX-sensitive ASs (Table 3) (Heuermann et al., 2016) (though fewer than HCN2 constitutive KO mice) and have decreased cortical and thalamic levels of HCN1–4 proteins. They also show a reduced depolarizing sag (but unchanged synaptic summation) of thalamic-projecting layer 5b pyramidal tract neurons and a decreased $I_h$ and increased cAMP-modulation in VB TC, but not NRT, neurons.

In summary, the available evidence in normal non-epileptic mice (Table 3) clearly indicates that 1) HCN2 channels in the VB have an anti-absence role whereas HCN4 channels in the VB either do not play an essential role in the generation of ASs or have a pro-absence effect; 2) there is a species difference with respect to the contribution of HCN1 channels to ASs, since HCN1 channels in rats contribute to ASs generation (though these data needs confirmation in a rat strain devoid of inherent ASs) whereas HCN1 channels in mice do not (though the absence of an AS phenotype could be explained by the compensatory increase of the tonic $GABA_A$ current of layer 5 neurons); 3) the role of different HCN channels in the neocortex has mostly not been studied; and 4) only one study has investigated the impact of genetically induced alterations of any HCN channel isoform on the $I_h$-dependent cellular properties of neurons in S1 layer 5 neurons where ASs initiate. In the next section, we will discuss the apparently contrasting results between these data in non-epileptic animals with different HCN isoform deletions and those obtained in well-validated genetic models of ASs.

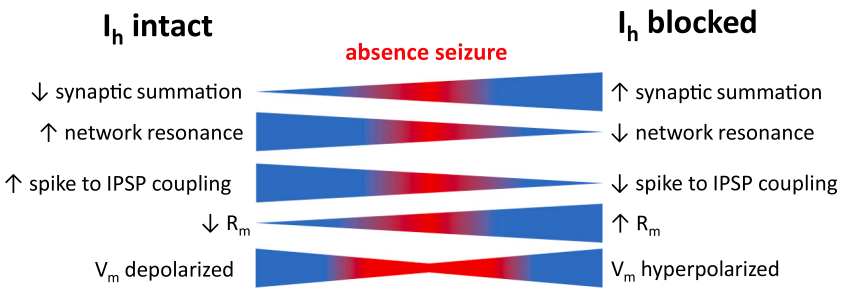


**Fig. 4.** Effect of $I_h$ on cellular and network properties leading to AS generation.
The schematic diagram shows how might develop when the amplitude of $I_h$ achieves a critical balance between its opposite effects on different cellular, synaptic and network properties. Thus, whereas a decreased $I_h$ will increase synaptic summation and $R_m$ leading to enhanced cellular and network excitability, it will at the same time hyperpolarize $V_m$, decrease network resonance and spike-to-IPSP coupling leading to reduced cellular and network excitability.

## 8. Role of HCN channels in ASs

### 8.1. *HCN1 isoform*

The following evidence suggests that the HCN1 isoform in the rat neocortex may have an anti-absence role: a) HCN1 proteins are expressed at higher levels in the neocortex (compared to HCN2–4) in normal non-epileptic animals (Notomi and Shigemoto, 2004), b) layer 5/6 pyramidal neurons in the CIN of WAG/Rij rats have decreased expression of HCN1 and reduced $I_h$-related properties whereas HCN2–4 isoform levels are unaltered (Blumenfeld et al., 2008; Kole et al., 2007; Strauss et al., 2004), c) HCN1 constitutive KO rats show ETX-sensitive ASs (Nishitani et al., 2019) (though this result should be confirmed in a rat strain that as no constitutive ASs), and d) the *HCN1* loss-of-function mutation identified in a child with CAE (Marini et al., 2018).

The lack of spontaneous ASs in HCN1 constitutive KO mice (Nolan et al., 2003; Santoro et al., 2010) might be due 1) to species differences, 2) the increased expression of HCN3 in both cortex and thalamus of these mice (Nolan et al., 2003; Santoro et al., 2010), 3) compensatory changes in thalamic $I_h$-related properties (that were not tested), and/or more importantly 4) the compensatory increase in the tonic $GABA_A$ current of S1 layer 5 pyramidal neurons that leads to an unaltered synaptic summation (Chen et al., 2010). Notwithstanding, a potential pro-absence role of the HCN1 isoform cannot at this stage be fully excluded since the whole-brain and forebrain-selective HCN1 KO mice were not tested for their sensitivity to pharmacologically induced ASs.

### 8.2. *HCN2 isoform*

The following evidence suggests that the HCN2 isoform in the mouse and rat thalamus may have an anti-absence role: a) the higher expression of HCN2 proteins in the thalamus (compared to HCN1, 3 and 4) of normal non-epileptic animals (Notomi and Shigemoto, 2004), b) the occurrence of spontaneous ASs in VB-selective HCN2 KO mice (Ludwig et al., 2003), c) the presence of spontaneous ASs in adult VB-selective HCN2 KD mice (Hammelmann et al., 2019), and d) the decreased cAMP-sensitivity in VB TC neurons of both GAERS and WAG/Rij rats (Budde et al., 2005; Cain et al., 2015; Kanyshkova et al., 2012; Kuisle et al., 2006). However, the contribution of the cortical HCN2 isoform to ASs remains unclear, since the $I_h$-related properties of the CIN layer 5 pyramidal neurons of any of the above HCN2-transgenic mice were not investigated.

### 8.3. *HCN4 isoform*

The following evidence suggests that the thalamic HCN4 isoform may either not significantly contribute to the expression of ASs or have a pro-absence effect: a) the unaltered HCN4 levels in the neocortex and thalamus of GAERS and WAG/Rij rats (Blumenfeld et al., 2008; Kanyshkova et al., 2012; Kuisle et al., 2006; Strauss et al., 2004), b) the lack of spontaneous ASs in HCN4 constitute KO mice (Zobeiri et al., 2019), and c) the lack of ASs in adult mice with VB-selective KD of the HCN4 isoform (Hammelmann et al., 2019). Taken together, the unchanged and decreased $I_h$ in CIN layer 5 pyramidal neurons of the HCN4 constitute KO mice (Zobeiri et al., 2019) and of the WAG/Rij rats (Kole et al., 2007), respectively, supports the critical role of HCN channels in these cortical neurons. Notably, the markedly decreased $I_h$ of VB TC neurons of the HCN4 constitutive KO mice (Zobeiri et al., 2019) indicate that this isoform makes a substantial contribution to thalamic $I_h$ despite its lower thalamic expression (compared to HCN2). Regrettably, the expression of the HCN1–4 isoforms and $I_h$-related properties in both the cortex and thalamus of the VB-selective HCN4 KD mice and the sensitivity of these mice and the HCN4 constitutive KO mice to pharmacologically induced ASs were not tested, making it difficult to draw a firm conclusion on the anti- or pro-absence role of the HCN4 isoform.

In sharp contrast with a potential anti-absence role of HCN1 and HCN2 isoforms, however, ASs are abolished and/or reduced when pan-HCN blockers and *sh*RNA (in GAERS rats and STG mice, respectively) are injected systemically, in the CIN or VB (David et al., 2018; Iacone et al., 2021) indicating that HCN1–4 channels in the whole brain, the cortex and the thalamus are essential for the full expression and maintenance of ASs.

A possible explanation of these contradictory results might be the effect of ivabradine and ZD7288 on other voltage-dependent channels: indeed, ZD7288 is known to affect both $Na^+$ and T-type $Ca^{2+}$ channels, at low and very high micromolar concentrations, respectively (Felix et al., 2003; Sánchez-Alonso et al., 2008; Wu et al., 2012). However, no changes in the action potentials of TC neurons were observed during similar microdialysis applications of ZD7288 in Wistar rats (David et al., 2018). Another explanation might be the presence of compensatory mechanisms in the transgenic mice, even in adult mice with viral transfection, since it is known that changes in thalamic HCN channel expression and function are observed as early as 7 days following an experimental cortical stroke (Paz et al., 2013). Moreover, after 5 days after of cuprizone treatment, a decrease in both ASs, HCN2 and HCN4 expression and $I_h$ as well as an increase in theta waves are observed in TC neurons of C3H/HeJ mice (Chaudhary et al., 2022). A similar scenario may be true for $I_h$ of the CIN layer 5 neurons that, regrettably, was not measured in any of the HCN1, HCN2 and HCN4 KO mice (except the HCN4 constitutive KO mice). A third potential reason to explain this discrepancy may be that the function of other channels, known to interact with HCN channels, is also abnormal in the AS models and/or the HCN isoform transgenic mice. Specifically, mGluR4 is co-localized with HCN2 in NRT neurons and has been shown to be critical for the GABAergic modulation of CT network synchronization (Snead et al., 2000). Moreover, in cortical pyramidal neurons HCN channels modulate Kir2 channels and $I_{Kleak}$ to shape synaptic summation (Day et al., 2005), and their interaction with the M-current results in a paradoxical effect on the amplitude of EPSPs in CA1 pyramidal neurons (George et al., 2009). Finally, the $I_h$ modulation of both long-term potentiation and depression shown in hippocampal neurons (Brager and Johnston, 2007; Fan et al., 2005) may also be present in cortical neurons and thus affect

their excitability during AS ictal activity. Thus, it would be interesting to systematically investigate $I_h$-related properties in S1 layer 5, VB and NRT neurons of the single- mutation models of ASs, *i.e.* STG, LH and TG mice.

## 9. How do HCN channels modulate the ictal firing of ASs?

We have a clear understanding of how a decreased cortical HCN channel function (mainly HCN1) contributes to the increased excitability and burst firing of single layer 5/6 pyramidal neurons of the CIN in AS model (*i.e.*, the WAG/Rij rats), *i.e.* increase synaptic integration and backpropagating action potentials leading to enhanced bursting probability (Kole et al., 2007). Whether this applies to other AS models and HCN transgenic mice, remains to be demonstrated. In this respect, it is unfortunate that the cellular and synaptic functions of S1 layer 5 neurons of the HCN transgenic mice (except the HCN4 constitutive KO mice) have not been investigated. However, HCN channels have also been shown to be essential for the expression of the neocortical network resonance that is important for the generation of theta waves (Ness et al., 2018; Stark et al., 2013), the frequency range of which covers the SWD frequency of rat (6–9 Hz) and particularly mice (4–6 Hz). The decreased $I_h$ of S1 layer 5 pyramidal neuron dendrites (Kole et al., 2007) can have a pro-absence effect (by increasing synaptic summation), but also an anti-absence effect (by reducing the resonance underlying the paroxysmal oscillation) (Fig. 4). Indeed, decreasing neocortical $I_h$ with a pan-HCN blocker injected in the GAERS CIN shifts the peak of the EEG theta frequency band toward lower frequencies during ictal and interictal periods (see Fig. 2C in Iacone et al., 2021). In contrast, injection of a pan-HCN blocker in the thalamus does not result in a change in the peak frequency of SWDs. (David et al., 2018). Moreover, recent studies in non-anaesthetized AS models have revealed substantial variability in the firing activity of cortical pyramidal cells and interneurons during ASs (McCafferty et al., 2018, 2023; Meyer et al., 2018). Therefore, understanding the cellular and synaptic processes by which HCN channels in various types of cortical interneurons in S1 deep layers tune and/or control SWDs expression is crucial. Without this information, it is difficult to propose a comprehensive mechanistic scenario of how the abnormal cortical HCN channel function in the CIN contributes to the paroxysmal network dynamics that initiates and maintains ictal activity during ASs.

All studies (listed in Tables 2 and 3) that have investigated the cellular and synaptic mechanisms by which induced or inherent alterations of thalamic HCN channels contribute to the ictal firing during ASs have used spontaneous or electrically evoked oscillations containing T-type $Ca^{2+}$ channel bursts in brain slices as a proxy for the paroxysmal activity of TC neurons during ASs, an approach based on the original *in vitro* model of an intrathalamic SWD pacemaker driven by rhythmic recurrent firing between TC and NRT neurons (von Krosigk et al., 1993). This model, however, does not reproduce the true ictal firing of thalamic neurons during ASs since 1) in the intact brain the membrane potential of TC and NRT neurons is more depolarized than in brain slices and thus only rarely are T-type $Ca^{2+}$ channel bursts observed *in vivo* during spontaneous SWDs in neurolept-anaesthetized GAERS rats (Charpier et al., 1999; Pinault et al., 1998, 2006), 2) the ictal firing of TC neurons in freely moving GAERS rats during SWDs is decreased (Fig. 3C) and mostly show single action potentials and few T-type bursts mainly at the start of an AS (Atherton et al., 2023; McCafferty et al., 2018, 2023), and 3) the ictal firing of NRT neurons during SWDs in freely moving GAERS is driven by the corticofugal input and not by the feedback excitation of TC neurons (Fig. 3D) (McCafferty et al., 2018). Thus, it is unlikely that the main HCN channel contribution to the ictal firing of TC neurons will be the repolarizing sag that follows the few T-type $Ca^{2+}$ bursts observed *in vivo*, nor would the termination of SWDs involve intracellular $Ca^{2+}$ accumulation shifting $I_h$ activation in TC neurons (Lüthi and McCormick, 1998). Notwithstanding, a role for HCN channels in the control of $V_m$ and $R_n$ of thalamic neurons cannot be excluded, in particular in NRT dendrites that have high HCN channel levels (Abbas et al., 2006) and are critical for the integrative capability of these neurons (Connelly et al., 2017). Moreover, as we suggested for the neocortical network, it is possible that HCN channels play a role in the resonant output of TC and NRT assemblies, whose firing during ASs is mainly determined by the activity of the neocortical afferents rather than their intrinsic pacemaker properties (McCafferty et al., 2018). This would also contribute to the marked reduction of ASs by a pharmacological block of thalamic HCN channels (David et al., 2018; Iacone et al., 2021).

## 10. Conclusions

Based on the available evidence, it is reasonable to conclude that HCN channels do indeed play a critical role in ASs but the detailed processes underlying their action are not yet fully understood. As indicated previously, achieving this goal is hampered by the many interactions between HCN and other voltage-gated channels and the opposite effects of HCN channels on different cellular, synaptic and network properties (Fig. 4). Indeed, whereas a decreased $I_h$ will increase synaptic summation and $R_m$ leading to enhanced cellular and network excitability, it will at the same time hyperpolarize $V_m$, decrease network resonance and spike-to-IPSP coupling leading to reduced cellular and network excitability. Thus, we propose that a critical range of $I_h$ values underlies the generation and maintenance of ASs (Fig. 4).

The following may aid in building a comprehensive mechanistic picture of HCN channels in ASs:

1) to conclusively confirm the role of the HCN1 isoform in the neocortex, the following experiments are suggested: a) KO of the HCN1 isoform in a rat strain devoid of ASs; b) sensitivity of HCN1 KO mice to drug-induced ASs, c) selective cortical- and thalamic ablation of HCN1 channels in mice and rats, and d) expression and function of HCN1 channels in layer 5/6 of the CIN of GAERS rats and mice AS models;
2) to confirm the role of HCN2 isoform, the following experiments are suggested: a) selective KD of the HCN2 isoform in the CIN of normal non-epileptic animals and b) CIN-selective KD of the HCN2 isoform in mouse and rat models of ASs.
3) to confirm the role of the HCN4 isoform, future experiments should investigate: a) the effect of CIN-selective *HCN4* deletion in normal animals and in mouse and rat AS models, and b) changes in $I_h$ and $I_h$-related properties in the cortex and thalamus of VB-selective HCN4 KD mice and mice AS models;
4) to confirm the role of $I_h$ in the AS-linked resonance, future experiments should investigate: a) the $I_h$ contribution to the resonance output of thalamic neurons at the depolarizing membrane potentials observed *in vivo*, b) the role of $I_h$ in the summation of excitatory and inhibitory synaptic potentials synaptic potentials of TC and NRT neurons, and c) the overall role of HCN channels using biophysical and dynamical computer models of the CT network where SWDs are driven by the firing of cortical layer 5/6 neurons (Dervinis and Crunelli, 2022).

Notwithstanding these missing mechanistic links, there is substantial evidence to support the notion that HCN channels blockers may provide a novel therapeutic approach for the treatment of ASs, and the development of HCN isoform-selective drugs will greatly contribute to current research on the role for these channels in ASs generation and maintenance as well as offer new potential clinical applications.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Data availability

Not applicable since this is review paper.

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